ENDOREPELLIN: METHODS AND COMPOSITIONS FOR INHIBITING ANGIOGENESIS

GOVERNMENT RIGHTS IN THE INVENTION

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FIELD OF THE INVENTION

The present invention relates to the fields of tumor biology and molecular biology, and to a method of treating angiogenesis-related diseases or conditions and, more particularly, to the inhibition of neovascularization and tumor growth and metastasis *in vivo*.

BACKGROUND OF THE INVENTION

Perlecan is a modular proteoglycan that participates in the formation and maintenance of basement membranes in various organs (1,2). It is a major heparan sulfate proteoglycan (HSPG) secreted by endothelial cells and is a potent inhibitor of smooth muscle cell proliferation, a biological function mediated by perlecan's block of fibroblast growth factor 2 (FGF2) activity (13) and Oct-1 gene expression (14). Perlecan has been shown to play a critical role in regulating the vascular response to injury *in vivo* (17).

Angiogenesis is one of the most important events in tumor progression and is greatly influenced by cell-matrix interactions taking place at the surface of the endothelial cells and at the tumor-matrix boundaries (31). HSPGs act as depots for pro- and anti-angiogenic factors and, in concert with members of the FGF and vascular endothelial growth factor (VEGF) families and their receptors, modulate various aspects of angiogenesis (9). Interestingly, various angiogenesis

inhibitors have been found to be proteolytically-processed forms of basement membrane collagens types IV, XV and XVIII, the latter two being chondroitin and HSPGs, respectively (32).

The invention presented herein describes a potent inhibitor of angiogenesis: the carboxyl terminus domain, domain V, of perlecan. This fragment, "endorepellin," inhibits angiogenesis and is active at nanomolar concentrations. Endorepellin interferes with endothelial cells' adhesive properties for various substrata, including, but not limited to, fibronectin and fibrillar collagen. Endorepellin, thus, represents a novel anti-angiogenic tool for the treatment of diseases or conditions associated with movement, migration and adhesion of cells, including diseases that involve angiogenesis such as, but not limited to, tumor metastasis and growth. The present invention provides endorepellin, analogs and fragments thereof, for use in inhibiting angiogenesis.

ABBREVIATIONS

"HSPG" means "heparan sulfate proteoglycans"

"HUVEC" means "human umbilical vein endothelial cells"

"CAM" means " chicken chorioallantoic membrane

"FGF" means "fibroblast growth factor"

"VEGF" means "vascular endothelial cell growth factor"

"LG" means "laminin-type G"

"EG" means "epidermal growth factor-like"

DEFINITIONS

"patient" as used herein can be one of many different species, including but not limited to, mammalian, bovine, ovine, porcine, equine, rodent and human.

"analog" as used herein is a derivative or modification of the native sequence. One skilled in the art may prepare such analogs wherein the native sequence is modified by resultant single or multiple amino acid substitutions,

additions or deletions. All such modifications resulting in a derivative of endorepellin are included within the scope of the invention, provided that the molecule retains angiogenesis-inhibiting activity.

"substantial sequence homology" means at least approximately 60% homology between the amino acid residue sequence in the endorepellin analog or derivative sequence and that of endorepellin sequence.

"anti-angiogenesis activity" and "angiogenesis-inhibiting activity" refers to the ability of a molecule to inhibit the growth of blood vessels.

"angiogenesis-mediated disease" refers to the unregulated growth of new blood vessels that causes a disease or exacerbates an existing condition.

DESCRIPTION OF THE DRAWINGS

Figure 1. Perlecan domain V (endorepellin) binds to the anti-angiogenic factor endostatin. **a**, Agarose gel showing the 1.7 kb cDNA strongly interacting with endorepellin, obtained from the BglII digestion of clone A3. Complete sequence of A3 clone revealed the C terminus of type XVIII collagen. **b**, Schematic representation of the human α chain of type XVIII collagen. The triple-helical and non-triple helical domains are indicated by rods and blue boxes, respectively. The C-terminal endostatin fragment is highlighted in orange. The beginning of the clone A3 sequence is shown (NCBI accession # AF018082). **c**, Growth and β-galactosidase activity triggered by the interaction of endorepellin with collagen type XVIII fragment compared to the positive (p53 and T-antigen) and negative control (lamin and T-antigen). **d**, Co-immunoprecipitation of collagen XVIII (clone A3) and endorepellin following *in vitro* transcription/translation using [^{35}S]methionine as the labeled precursor. Endorepellin (lane 1) and collagen XVIII (lane 2) are mixed in equimolar amounts and co-immunoprecipitated with either anti-hemagglutinin (α-HA) (lane 3) or no antibody. **e**, Co-immunoprecipitation of endostatin with endorepellin. Domain III (lane 1), endorepellin (lane 2) and endostatin (lane 3) were generated by *in vitro* transcription/translation using [^{35}S]methionine

as the labeled precursor. Endostatin was mixed with either domain III (lane 4) or endorepellin (lane 5) and immunoprecipitated with anti-hemagglutinin (α-HA) antibody. **f**, Schematic representation of domain V and various deletion mutants. Orange ovals indicate laminin-type G modules (LG), whereas blue rectangles indicate EGF-like (EG) modules. The growth is indicated by semi-quantitative assessment with maximal growth at +++. The numbers within parentheses designate the amino acid position based on the mature protein core. **g**, Representative α- and β-galactosidase assays of various deletion mutants, as indicated; pGB53/pGADT was the positive control.

Figure 2. Endorepellin is a powerful anti-angiogenic factor. **a**, Purification of endorepellin from media conditioned by 293-EBNA cells expressing the 81 kDa

endorepellin tagged with His6. Coumassie-stained SDS-PAGE (left) and Western

immunoblotting with anti-His6 antibody (right) of negative control media (lanes 1 and 4), flow through (lanes 2 and 5), and 250 mM imidazole eluate (lanes 3 and 6). **b** and **c**, HUVEC migration assays through fibrillar collagen using 10 ng/ml VEGF as a chemotactic inducer and preincubation the HUVECs for 30 min with various concentrations of endostatin (ES) and endorepellin (ER). Serum free medium (SFM). **d**, CAM assays three days after the application of sponges containing VEGF (1 ng), VEGF (1 ng)+ endorepellin (400 ng), or buffer alone. Scale bar, 1 mm.

Figure 3. Endorepellin, but not endostatin, blocks endothelial tube formation induced by fibrillar collagen. **a-d**, Gallery of light micrographs capturing the time course production of HUVEC tube-like formation in fibrillar collagen containing either buffer (Control), endorepellin, endostatin, or both at the designated concentrations. In this assay, 4 x 10^5 cells are incubated for 24 hr and pictures are taken at various intervals as indicated in the top margins. Scale bar, 250 μm.

Figure 4. Biological consequences of endostatin/endorepellin interaction. **a** and **b**, HUVEC migration assays through fibrillar collagen using 10 ng/ml VEGF as a chemotactic inducer and preincubation the HUVECs for 30 min with various concentrations of endostatin (ES), endorepellin (ER), or various combinations as indicated. The values are presented as the percentage of maximal stimulation induced by VEGF alone, arbitrarily set at 100%. Panel a is the summary of three independent experiments run in quadruplicates, mean ±SE. The values in panel b derive from an additional experiment run in quadruplicate, mean ± SE. Serum free medium (SFM).

Figure 5. Endorepellin is counter-adhesive for endothelial, fibrosarcoma and colon carcinoma cells. **a**, HUVEC adhesion to increasing concentrations of various substrata including fibronectin (✭), BSA (↨), or endorepellin (θ). For each point, 5 X10^4 cells are seeded on the various substrata. After 1 h, adherent cells are washed, stained with crystal violet and solubilized in O.1% Triton X-100, and absorbance monitored at 600 nm. The number of attached cells is proportional to the absorbance. About 80% of the total cells are attached in the plateau region of the fibronectin curve. The values represent the mean ±SE (n=4). **b**, Gallery of light micrographs of crystal violet-stained HUVECs adhered to 50 nM fibronectin following incubation for 1 hr with endorepellin at the indicated concentrations. Scale bar, 125 μm. **c** and **d**, Adhesion assays for HT1080 fibrosarcoma and WiDr colon carcinoma cells, respectively, on fibronectin (✭), BSA (↨), or endorepellin (θ) substrata. The conditions are identical to those described in panel a. The values represent the mean ±SE (n=4). **e** and **f**, Displacement curves employing increasing concentration of either endorepellin or endostatin, respectively. The calculated IC_{50} for HT1080 and WiDr was 110 and 40 nM, respectively. The values represent the mean ± SE (n=4).

DESCRIPTION OF THE INVENTION

Angiogenesis, the process by which new blood vessels are formed, is essential for normal bodily activities including reproduction, development and wound repair. Although the process is not completely understood, angiogenesis is believed to involve a complex interplay of molecules, which regulate the growth of endothelial cells (the primary cells of capillary blood vessels). Under normal conditions, these molecules appear to maintain the microvasculature in a quiescent state (i.e. one of no capillary growth) for prolonged periods which may last for as long as weeks or, in some cases, decades. When necessary (such as during wound repair), these same cells can undergo rapid proliferation and turnover within a 5 day period.

Although angiogenesis is a highly regulated process under normal conditions, many diseases (characterized as angiogenic diseases) are driven by persistent unregulated angiogenesis. Otherwise stated, unregulated angiogenesis may either cause a particular disease directly or exascerbate an existing pathological condition. For example, growth and metastasis of solid tumors are dependent on angiogenesis. It has been shown, for example, that tumors which enlarge to greater than 2 mm must obtain their own blood supply and do so by inducing the growth of new capillary blood vessels. Neovascularization of a tumor enables the metastatic spread of tumor cells by providing a route of escape for the metastatic cells and nurturing the tumor by providing a growth-conducive environment. Another example is ocular neovascularization, a disease that has been implicated as the most common cause of blindness and dominates approximately twenty distinct eye diseases. In certain existing conditions, such as arthritis, newly formed capillary blood vessels invade the joints and destroy cartilage. In diabetes, new capillaries formed in the retina invade the vitreous, bleed, and cause blindness.

Many other diseases are driven by persistent unregulated angiogenesis. The diseases or conditions treatable by the present invention include, but are not limit to, primary tumor growth, tumor invasion or metastasis, hemangioma, leukemia, atherosclerosis, post-balloon angioplasty, myocardial angiogeneisis, plaque neovascularization, vascular restenosis,

neointima formation following vascular trauma, vascular graft restenosis, fibrosis associated with a chronic inflammatory condition, lung fibrosis, chemotherapy-induced fibrosis, wound healing with scarring and fibrosis, psoriasis, deep venous thrombosis, corneal diseases, ovulation, menstration, placentation, or any other disease or condition which is mediated by angiogenesis. In addition, an important new medical method for birth control, wherein an effective amount of endorepellin is administered to a female such that uterine endometrial vascularization is inhibited and embryo implantation does not occur or is not sustained, is also contemplated by the present invention.

Angiogenesis inhibitors

Angiogenesis inhibitors currently under development for use in treating angiogenic diseases have many disadvantages. Suramin, for example, is a potent angiogenesis inhibitor but causes severe systemic toxicity in humans at doses required for antitumor activity. Compounds such as retinoids, interferons and anti-estrogens are safe for human use but have weak anti-angiogenic effects. Thus, there is a need for compounds useful in treating angiogenic diseases in mammals. More specifically, there is a need for angiogenesis inhibitors which are safe for therapeutic use and which exhibit selective toxicity with respect to the pathological condition, such as by selectively inhibiting the proliferation of cancer cells while exhibiting no, or a low degree of toxicity to normal (ie. non-cancerous) cells. The present invention provides such a compound, endorepellin, to treat angiogenic-mediated diseases in mammals.

Furthermore, certain types of tumors are more amenable to therapy than others. Such tumors include, soft tissue tumors such as tumors of the blood. The reason for the increased efficacy in treatment regimens with respect to these tumors is their greater accessibility to chemotherapeutic agents. In contrast, it is much more difficult for most chemotherapeutic agents to reach all of the cells of a solid tumor mass.

Solid tumors rely on the generation of new blood vessels in order for the nutrients to reach the cells within the tumor. Thus, the present invention

provides a novel therapeutic approach to the treatment of solid tumors wherein the generation of new blood vessels within the tumor, rather than the tumor cells themselves, is the target. This treatment is not likely to lead to the development of resistant tumor cells. Moreover, inhibition of angiogenesis leads to an amplification of the anti-tumor effect since many tumor cells rely on a single vessel for their nutrients.

Methods

Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, cell culture, and transgene incorporation (e.g., electroporation, microinjection, lipofection). The enzymatic reactions, oligonucleotide synthesis, and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are performed according to conventional methods in the art and various general references that are provided throughout this document. The procedures therein are well known in the art, some of which are provided for the convenience of the reader.

Primary culture and cell lines.

Primary cultures of HUVECs are prepared from fresh umbilical cords and cultured on collagen-coated flasks in M199 or M200 medium supplemented with 10% FBS, 50 μg/ml heparan and endothelial cell growth supplement, isolated from bovine hypothalami. Only passages 4 to 8 are used. A431 squamous carcinoma cells, HT1080 fibrosarcoma, WiDr colon carcinoma, MCF7 breast carcinoma, and M2 mouse melanoma cells are obtained from American Type Culture Collection (Manassas, Virginia).

Yeast two-hybrid screening and co-immunoprecipitation.

To reduce the number of false positives, the Matchmaker GAL4 two-hybrid system 3 (Clontech, Palo Alto, CA), which adopts three independent reporter genes for the selection, His, Ade and α or β-galactosidase, is used. A keratinocyte library constructed in the pACT2 vector is amplified and the plasmids are extracted employing the NucleoBond AX Giga plasmid

purification kit (both the vector and kit are from Clontech). Endorepellin is subcloned into the pGBKT7 vector using PCR generated fragments as previously described (44), and is used as a bait to screen ~5 x 10^6 cDNAs. The yeast clones growing in selective medium are re-plated in quadruple minus plates containing X-gal. The plasmids from the blue colonies are isolated, expanded by subcloning in DH5α bacterial cells, and analyzed on agarose gels after restriction digestion with BglII. The inserts are identified by automatic sequencing.

Seven deletions of perlecan domain V are obtained by PCR, including suitable restriction sites to allow unidirectional ligation into the pGBKT7 vector (44). The various proteins are *in vitro* transcribed and translated in presence of [^{35}S]methionine (ICN Pharmaceuticals, Costa Mesa, California) employing the TNT® reticulocyte lysate system (Promega, Madison, Wisconsin). One μg of the pGBKT7 or pGADT7 constructs is employed and the reactions are incubated for 90 min at 30°C. Aliquots are subjected to co-immunoprecipitation with affinity-purified, anti-hemagglutinin (αHA) rabbit polyclonal antibodies (Clontech). The immune-complexes are captured with protein A/G agarose beads (Pierce, Rockford, Illinois). The beads are washed three times with HNTG buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% triton X100, 10% glycerol, 200 mM Na_2VO_4, 20 mM NaF and an EDTA-free protease inhibitors cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The bands are separated in polyacrylamide gels, fixed in the presence of AMPLIFY™ (Amersham Pharmacia Biotech, Uppsala, Sweden), dried under vacuum and exposed to KODAK films.

<u>Expression and purification of recombinant endorepellin.</u>

The pCEP-Pu vector bearing the sequence of the BM40 signal peptide is used to transfect by electroporation ~10^7 human kidney cells (293-EBNA) expressing the Epstein-Barr virus nuclear antigen (EBNA)-1. Mass cultures are selected in media containing 250 μg/ml G418 and 500 ng/ml puromycin. The confluent cells are allowed to express the recombinant protein in serum

free for 48 hr. The conditioned media are concentrated in a dialysis bag with PEG and dialyzed in sonication buffer (12.5 mM Na_3PO_4, 75 mM NaCl, pH= 8.0) and further purified employing the Ni-NTA resin and eluted with 250 mM imidazole. The fractions containing the recombinant protein are dialyzed against 10 mM Hepes, 150 mM NaCl and 2 mM EDTA and further concentrated. In all the purification steps phenylmethylsulfonyl fluoride (2 mM) and N-ethylmaleimide (2 mM) are the protease inhibitors. Using this procedure, 5-10 mg of endorepellin/L is routinely purified from conditioned medium. ELISA and immunoblotting with anti-domain V (16) or Penta-His (QIAGEN, Valenica, California) monoclonal antibodies is performed as described before (24).

<u>Endothelial cell migration, tube formation and chorioallantoic membrane (CAM) assays</u>.

A 48-well Boyden chamber (Neuroprobe Inc., Gaithersburg, Maryland) is used for migration assays employing HUVECs. VEGF165 (R&D Systems, Minneapolis, Minnesota) is used as a chemo-attractant in M199 (Life Technologies, Carlsbad, California) containing 0.1% BSA. Cells migrated through eight-micron nucleopore polyvinylpyrrolidine-free polycarbonate filters (Corning, Cambridge, Massachusetts) pre-coated for 48 hr with 100 μg/ml collagen type I (Collaborative Biomedical Products, Bedford, Massachusetts) in 0.2 N acetic acid and air dried. The endothelial cells, trypsinized and re-suspended in M199 medium, are pre-incubated for 1 h with different concentrations of endorepellin and/or endostatin (Calbiochem-Novabiochem, San Diego, California). The cells are allowed to migrate through the filter for 6 h at 37°C with 5% CO_2. Every experiment is performed in quadruplicate, after each incubation, the filters are washed, fixed, stained with Diff-Quick stain (VWR Scientific Products, Bridgeport, New Jersey), and counted in total using conventional microscopy.

For *in vitro* tube-like formation, 12-well cell culture dishes are coated with 100 µg/ml collagen type I in sterile 10 mM acetic acid. HUVECs seeded for 18 hr, and then covered with a second layer of collagen (41). Cultures are incubated until gels had solidified, typically 15-30 min, and then given 1 ml of media containing the various test agents and control substances.

For the CAM assays, fertilized White Leghorn chick eggs are incubated at 37°C. After three days of incubation, ~ 3 ml of albumin was removed to detach the CAM. A small square window is then opened in the eggshell, which is sealed with tape and the eggs are returned to the incubator. At day 9, a ~ 1 mm^3 Gelfoam sterile sponge Gelfoam (Pharmacia, North Peapak, New Jersey) is placed on the chorioallantoic membranes and various test factors are applied including VEGF, endorepellin or buffer alone. The development of blood vessels is monitored by light microscopy up to 12 days.

<u>Cell adhesive and counter-adhesive assays</u>.

HUVECs and various tumor cells lines, including WiDr, A431, MCF7 and M2 cells, are tested for adhesion to various substrata including fibronectin, collagen type I, BSA, endorepellin or endostatin as plastic-immobilized substrata using dose-response profiles (coating concentrations of 10-180 nM). 5 x 10^5 cells are plated in quadruplicate wells and, after 1 hr of incubation, adherent cells are washed, fixed in 1 % glutaraldehyde for 10 min, stained with crystal violet, lysed with 0.2% Triton X-100, and assayed by a colorimetric test (33). The crystal violet concentration, which is proportional to the cell number, is estimated at A_{600}.

The anti-adhesive assays are performed in a similar way. About 40µg/ml fibronectin are used to coat individual plates. After blockage with 1% BSA, the cells are added to the wells in the presence of increasing concentration of endorepellin or endostatin. After 1 h of incubation, the wells are

treated as above. Additional details are provided in the legends to the figures and in the methods (*supra*).

Endorepellin, analogs and fragements thereof

The term " endorepellin " refers to a protein that is approximately between 210 amino acids and 705 amino acids in size. The term endorepellin also includes fragments of the 705 amino acid protein and modified proteins and peptides that have a substantially similar amino acid sequence, and which are capable of inhibiting angiogenesis. Silent substitutions of amino acids, wherein the replacement of an amino acid with a structurally or chemically similar amino acid does not significantly alter the structure, conformation or activity of the protein, are well known in the art. For example, one polar amino acid(s), such as threonine, may be substituted for another polar amino acid(s), such as serine. Such silent substitutions are intended to fall within the scope of the appended claims. It will be appreciated that the term "endorepellin" includes shortened proteins or peptides wherein one or more amino acids is removed from either or both ends of endorepellin, or from an internal region of the protein, yet the resulting molecule retains angiogenesis-inhibiting activity. The term "endorepellin" also includes lengthened proteins or peptides wherein one or more amino acids is added to either or both ends of endorepellin, or to an internal location in the protein, yet the resulting molecule retains angiogenesis inhibiting activity.

Thus, the present invention contemplates amino acid residue sequences that have substantial sequence homology to endorepellin such that those sequences demonstrate like biological activity. It is well known in the art that modifications and changes can be made to a peptide without substantially altering the biological function of that peptide. For example, alterations to endorepellin peptide fragments may enhance the peptide's potency or stability to enzymatic breakdown. Such contemplated sequences include those analogous sequences characterized by a change in amino acid residue sequence or type wherein the change does not alter the fundamental nature and biological activity of the aforementioned endorepellin.

Generation of antibodies to endorepellin and uses thereof

According to the invention, endorepellin, its fragments or derivatives, or analogs thereof, may be used as an immunogen to generate antibodies that recognize such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and Fab expression library. In a specific embodiment, antibodies to endorepellin are produced.

Methods of producing endorepellin for generation of antibodies include, but are not limited to, recombinant DNA techniques, peptide synthesis wherein multiple fragments are synthesized and subsequently linked together to form the full length endorepellin, or proteolyic digestion of perlecan. These protocols are standard technology and well known to those of skill in the art.

Various procedures known in the art may be used for the production of polyclonal antibodies to endorepellin or derivatives or analogs thereof. For the production of antibody, various host animals can be immunized by injection with the native endorepellin, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward an endorepellin sequence or analog thereof, any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. Examples of such techniques include, but are not limited to, the hybridoma, as well as the trioma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique to produce human monoclonal antibodies. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, such as, but not limited to, ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies that recognize a specific domain of endorepellin, one may assay generated hybridomas for a product that binds to an endorepellin fragment containing such domain.

Diagnostic uses of antibodies

Anti-endorepellin antibodies may be generated (*supra*) and used to detect, prognose, diagnose, or monitor the treatment of angiogenesis-mediated diseases, such as cancer, by detecting the presence of endorepellin in patient samples. For detection of endorepellin sequences, a diagnostic kit of the present invention comprises, in one or more containers, an anti-endorepellin antibody that can be detectably labeled. In a different embodiment, the kit can comprise, in one or more containers, a labeled specific binding portion of an antibody. As used herein, the term "detectable label" refers to any label that provides directly or indirectly a detectable signal and includes, for example, enzymes, radiolabelled molecules, fluorescent molecules, particles, chemiluminesors, enzyme substrates or cofactors, enzyme inhibitors, or magnetic particles. Examples of enzymes useful as detectable labels in the present invention include, but are not limited to, alkaline phosphatase and horse-radish peroxidase. A variety of methods are available for linking the detectable labels to antibodies and include for example the use of a bifunctional agent, such as, 4,4'-difluoro-3,3'-dinitro-phenylsulfone, for attaching an enzyme, for example, horse radish peroxidase, to an antibody. The attached enzyme is then allowed to react with a substrate yielding a reaction product that is detectable. The present invention provides a method for detecting endorepellin in a patient sample, comprising, contacting the patient sample with an anti-endorepellin antibody under conditions such that immunospecific binding occurs, and detecting or measuring the amount of any immunospecific binding by the antibody.

Patient samples are any sample from a patient thought to contain endorepellin. Samples include, but are not limited to, peripheral blood or

serum, urine, tissue sections, peritoneal fluid, cerebrospinal fluid, uterine fluid, mucus, etc.

Administration

This invention includes methods for inhibiting angiogenesis. By inhibiting angiogenesis, tumor metastasis is inhibited. In this method, a patient is administered an amount of endorepellin, its fragments or derivatives, or analogs thereof, effective to inhibit angiogenesis. The compound, or pharmaceutically acceptable salt thereof, is administered in the form of a pharmaceutical composition (*infra*).

Doses of the compounds include pharmaceutical dosage units comprising an effective amount of the peptide. By an effective amount is meant an amount sufficient to achieve a steady state concentration *in vivo* which results in a measurable reduction in any relevant parameter of disease, such as growth of primary or metastatic tumor, any accepted index of inflammatory reactivity, or a measurable prolongation of disease-free interval or survival.

In one embodiment, an effective dose is approximately between10-fold and 100-fold higher than the 50% inhibitory concentration (IC_{50}) of the compound.

The amount of active compound to be administered depends on the precise peptide or derivative selected; the disease or condition; the route of administration; the health and weight of the recipient; the existence of other concurrent treatment; if any, the frequency of treatment, the nature of the effect desired, for example, inhibition of tumor metastasis; and the judgment of the skilled practitioner. The precise dose to be employed is decided according to the judgement of the practitioner and each patient's circumstances. The following are examples of such doses and is not meant to limit the invention in any way.

The proteins and protein fragments with endorepelliin activity are provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations are administered by

standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the endorepellin, its fragments or derivatives, or analogs thereof, may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the endorepellin, its fragments, derivatives, or analogs thereof, is slowly released systemically. Osmotic minipumps also may be used to provide controlled delivery of high concentrations of endorepellin, its fragments, derivatives, or analogs thereof, through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor.

An exemplary dose for treating a patient with a tumor is an amount of up to about 100 milligrams of active compound per kilogram of body weight.

Typical single dosages of the peptide are between about 1 μg and about 100 mg/kg body weight. For topical administration, dosages are in the range of about 0.01-20% concentration of endorepellin, its fragments or derivatives, or analogs thereof. A total daily dosage in the range of about 10 milligrams to about 7 grams is exemplary for oral administration. The foregoing ranges are, however, exemplary, as the number of variables in regard to an individual treatment regimen is large, and considerable excursions from these recommended values are expected.

The therapeutic regimen with endorepellin, its fragments or derivatives, or analogs thereof, compounds of the invention produce an inhibitory effect on cell migration and invasion, on angiogenesis, on tumor metastasis and/or on inflammatory reactions in a patient having a disease or condition associated with undesired cell invasion, migration-induced proliferation, angiogenesis or metastasis (*supra*).

Endorepellin, its fragments, derivatives, or analogs thereof, of the present invention are useful for inhibiting angiogenesis when used alone or in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery,

radiation or chemotherapy and endorepellin, or analogs or derivatives thereof, administration, either concurrently or subsequently, to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

Pharmaceutical compositions

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of endorepellin, its fragments, derivatives, or analogs thereof, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes, but is not limited, to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition are sterile. The formulation suits the mode of administration.

The composition, if desired, also can contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In one embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition also may include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for xample, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the

composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The endorepellin, its fragments, derivatives, or analogs thereof, of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Results

Endostatin is a novel interacting partner for perlecan domain V/endorepellin

Using endorepellin as bait, a keratinocyte cDNA library is screened in the yeast two-hybrid system. One of the strongest interacting clones (clone A3) encoded the C-terminal portion of collagen type XVIII, containing the NC1 domain and the potent anti-angiogenic factor endostatin (**Fig. 1a** and **b**). Since endostatin inhibits endothelial cell proliferation and effectively arrests the growth of several tumors (28), and because perlecan and endostatin co-localize in most tissues (29, 30), it is reasoned that an interaction between these two proteins could occur *in vivo* and could play a role in tumor progression. Therefore, the collagen fragment is sub-cloned into the pGADT7 vector, and the interaction with endorepellin is once more tested with the two-hybrid system on a one-to-one basis. The growth of the cells in quadruple minus medium is comparable to that of the positive control (pGBKT7-53/pGADT7-T), as well as the blue color generated by α-galactosidase expression (**Fig. 1c**).

To corroborate the yeast interaction, collagen XVIII and endorepellin are transcribed and translated *in vitro*, resulting in the ~81 and ~65 kDa fragments, respectively (**Fig. 1d**). The two proteins are co-precipitated with an anti-hemagglunitin antibody which recognizes the oligopeptide epitope HA

present at the C-terminus of collagen XVIII (**Fig. 1d**). To map the site of interaction, a deletion fragment of NC1 domain is cloned into pGADT7 containing only endostatin. The NC1 domain is then transcribed *in vitro* and translated resulting in a 23 kDa band (**Fig. 1e, lane 3**). As a further control, perlecan domain III is transcribed and translated, producing a ~130 kDa band (**Fig. 1e, lane 1**). The results show that only endorepellin interacts with endostatin (**Fig. 1e, lane 5**). In contrast, domain III does not bind to endostatin (**lane 4**). These experiments are repeated three times with comparable results.

Endostatin interacts specifically with the LG2 module of endorepellin

To establish the precise location of this interaction, seven deletion mutants of of domain V/endorepellin are generated, Δ1-Δ7 (**Fig. 1f**). This domain consists of three laminin type G (LG1-LG3) modules separated by four EGF-like (EG1-EG4) modules in an arrangement highly conserved across species (1). Robust growth in quadruple minus media is observed in cells co-transformed with full-length endorepellin and endorepellin with two deletions, Δ1 and Δ5, which contain the LG2 module (**Fig. 1f**). These results are corroborated by α- and β-galactosidase assays (**Fig. 1g**). Further support for a true protein/protein interaction is growth in amino acid-deficient media, transcription of LacZ (α- and β-galactosidase) under the control of distinct GAL4 upstream activating sequences, and the subsequent ability of the co-transformant yeast strains to express functional galactosidase activity. Thus, the LG2 module of endorepellin is the specific site of endostatin binding.

Recombinant endorepellin is anti-angiogenic

Human recombinant endorepellin, generated in 293-EBNA cells, migrated on SDS-PAGE as a single band of the predicted ~81 kDa. The identity of this recombinant protein is further confirmed by immunoblotting with anti-His6 antibody (**Fig. 2a**) and ELISA, using a specific monoclonal antibody against domain V (12). To test the biological properties of endorepellin, VEGF-induced migration of human umbilical vein endothelial cells (HUVEC)

subcloned to passages 4-8 (31) is used. It is well established that the motility and vectorial migration of endothelial cells that occur with invasion is a fundamental component of angiogenesis (27,32). When VEGF is used in the lower chamber, there is a complete suppression of HUVEC migration through the membrane at 1-10 μg/ml (12-120 nM) endorepellin (**Fig. 2b**). Interestingly, endorepellin is more active than recombinant endostatin purified from *Pichia pastoris* yeast cells. Subsequent dilution experiments revealed that endorepellin is fully active at 0.5 μg/ml (6 nM) (**Fig. 2c**), with a calculated IC_{50} of 1.2 nM (± 0.1, n=11). In some preparations, endorepellin is active even at picomolar concentrations, similar to those reported for endostatin produced by 293-EBNA cells (31), the same cells used in this study. The experiments in **Fig. 2b and c** are repeated eleven times with various preparations of endorepellin, and a marked suppression of HUVEC migration is consistently found. In contrast to endostatin, the migratory response is not dependent on preincubation of the endothelial cells with endorepellin. In experiments where endorepellin is placed in the lower chambers of the invasion assay, similar inhibition of VEGF-induced migration is found.

Endorepellin blocks the angiogenic activity of VEGF (**Fig. 2d**), as determined by the chicken chorioallantoic membrane (CAM) assay. Thus, HUVEC migration is inhibited, with the subsequent decrease in angiogenesis *in vivo*. In the presence of VEGF, the characteristic spoke wheel-like vessel formation is induced towards the sponge. In the presence of endorepellin, the vessel sprouts are markedly reduced to a level comparable to the negative control. These experiments are repeated several times with identical results.

To further investigate the role of endorepellin in angiogenesis, HUVEC tube formation is utilized in a collagen matrix (33). The results show a capillary-like network formation in the control HUVECs (**Fig. 3a**), which is visible at 4 h and remained constant for up to 24 h. In contrast, endorepellin causes a complete block of tube-like formation (**Fig. 3b**), whereas no significant effects were obtained with endostatin (**Fig. 3c**). Surprisingly, endorepellin activity is counteracted by endostatin (**Fig. 3d**). It should be noted that the concentration of endorepellin used in these assays (μg/ml) is even lower than that used for the migration assays. In fact, while in the latter,

full inhibition is detected at 300-500 ng endorepellin/10^4 HUVEC (30-50 pg /cell), in the former, full inhibition is achieved at 10 μg endorepellin/4 x 10^5 HUVEC (25 pg/cell). Thus, the present invention reveals that endorepellin is a powerful blocker of angiogenesis in three independent assays commonly used to investigate angiogenesis.

Biological effects of endostatin/endorepellin interaction

To further investigate the interaction between endostatin and endorepellin, several VEGF-induced HUVEC migration experiments are performed in which the amount of endorepellin is kept constant while the amount of endostatin is proportionally increased. Two concentrations of endorepellin are used, 1.2 and 3.7 nM (100 and 300 ng/ml, respectively), that give suboptimal and optimal inhibition of HUVEC migration. When endostatin and endorepellin are concurrently present, there is a significant and dose-dependent inhibition of their activity (**Fig. 4a** and **b**). By plotting the percentage of migrated cells, derived from normalized data of five independent experiments against the increasing molar ratios of endostatin/endorepellin, it is evident that maximal inhibition is achieved at about 1:1 molar ratio; the inhibition subsequently declined (not shown). Thus, the interaction between endostatin and endorepellin leads to a marked attenuation of their anti-angiogenic activity.

Endorepellin has counter-adhesive properties for endothelial cells

A number of bioactive fragments of extracellular matrix proteins exhibit counter-adhesive activities, that is, they disrupt cell-matrix interactions (34). It has been previously shown that domain V of perlecan is adhesive for several cell lines vis á vis fibronectin, but not for others (35). To address this point, endorepellin is tested for its ability to mediate HUVEC adhesion. The result is a complete lack of HUVEC adhesion to either endorepellin or BSA, in contrast to a robust adhesion to fibronectin (**Fig. 5a**) or collagen type I. In competition experiments, in which HUVECs are challenged with increasing amounts of endorepellin, a progressive inhibition of HUVEC attachment occurs. Within minutes, the cells rounded up and began to detach in a dose-

dependent manner (**Fig. 5b**). Several experiments on fibrillar collagen or plastic are performed and endorepellin consistantly prevented HUVEC binding to either substratum, with IC_{50} values of 5-20 nM. In contrast, endostatin does not show any interference with endothelial cell attachment to either fibronectin or collagen I.

To verify that this anti-adhesive property of endorepellin is not limited to endothelial cells, HT1080 fibrosarcoma cells, which do not bind to murine domain V (35), and WiDr colon carcinoma cells (14) are tested. Endorepellin does not support adhesion of either HT1080 fibrosarcoma cells or WiDr colon carcinoma cells (**Fig. 5c and d**). Moreover, the specificity of endorepellin counter-adhesive properties is demonstrated by the efficient displacement of HT1080 and WiDr attachment to fibronectin using increasing concentrations of recombinant endorepellin (IC_{50} of 110 and 40 nM, respectively; **Fig. 5e**). In contrast, endostatin does not significantly affect the adhesion of either cell line (**Fig. 5f**).

Adhesion assays also are performed using fibronectin and two concentrations of collagen type I with A431 squamous carcinoma cells, which were previously shown to adhere to murine domain V (58% of the values with a mean attachment value of 52 ± 4 % (**Table 1**) were obtained). Mouse M2 melanoma and human MCF7 breast carcinoma cells are also analyzed for adhesion in the presence of endorepellin. The resultant attachment values are <5% for mouse M2 melanoma cells and 50 ± 7% for human MCF7 breast carcinoma cells (**Table 1**). Thus, endorepellin is a powerful anti-adhesive factor for endothelial cells and certain tumor cells.

Discussion

The present invention discloses screening, *in vivo,* for molecules that interact with the perlecan protein core. It is discovered that the cDNA encoding endostatin, the anti-angiogenic C-terminal fragment derived from collagen type XVIII (28), interacts strongly with the perlecan protein core. It previsously has been shown, using an *in vitro* cell-free system, that the entire perlecan binds to endostatin, presumably via the heparan sulfate chains (36,37). These results are confirmed herein. In addition, the present invention

shows that a distinct subdomain of perlecan protein core binds specifically to endostatin.

Using a battery of deletion mutants, the precise binding site of endostatin to perlecan is mapped to the second laminin-like G (LG) domain of perlecan domain V. Since perlecan and type XVIII collagen/endostatin co-distribute in basement membranes (29,30,36) and endostatin binds *in situ* to vascular basement membranes independently of heparan sulfate (38), domain V is a likely binding site for endostatin *in vivo*.

Using HUVEC migration assays the present invention reveals that the interaction between endostatin and domain V counteracts the anti-angiogenic effects of endostatin. Since perlecan domain V itself is a powerful anti-angiogenic factor it is named herein "endorepellin". Endorepellin is active at nanomolar concentrations and is a potent inhibitor of angiogenesis in three independent assays commonly used to study angiogenesis: endothelial cell migration through fibrillar collagen, collagen-induced capillary-like formation, and growth of blood vessels in the chorioallantoic membrane. Interestingly, the action of endorepellin is as strong as endostatin in inhibiting HUVEC migration. Endorepellin interferes with the adhesive properties of endothelial cells for various substrata, including, but not limited to, fibronectin and fibrillar collagen, and also is anti-adhesive for certain tumor cells derived from colon, neuroectoderm or mesenchyme. This is in agreement with previous studies showing anti-adhesive properties for perlecan in hematopoietic (24), mesangial (25) and smooth muscle (39) cells, and a role for perlecan in the suppression of growth and invasion in fibrosarcoma cells (40). However, while endorepellin inhibits tube formation and prevents binding to fibronectin and other substrata, endostatin does not.

Powerful angiogenesis inhibitors are proteolytically-processed forms of basement membrane collagens types IV, XV and XVIII, the latter two being chondroitin and HSPGs, respectively (41). Moreover, proteolytic remodeling of the extracellular matrix can expose cryptic sites within collagen type IV that are required for angiogenesis *in vivo* (42). Thus, it is likely that perlecan undergoes a similar proteolytic processing *in vivo*, thereby liberating endorepellin.

The modular nature of the perlecan protein core is particularly well suited for selective proteolysis (12,41,43) and subsequent release of peptides with biological activity. There are two lines of evidence that support this scenario. First, in 293-EBNA cells a natural proteolytic cleavage of endorepellin of ~25 kDa is detected which binds to the Ni-NTA column and is also reactive with the anti-His6 antibody, indicating that it represents LG3. A similar size band previously was shown to represent a proteolytic fragment of murine domain V generated by cleavage just before the beginning of LG3 (9,35). This protease-sensitive region, which starts with the sequence DAPGQY **(SEQ. ID. NO: 1)**, is completely conserved between mouse (3) and human (4), thus demonstrating that a specific cleavage of Asn-Asp bond (at position 3514/3515 and 4196/4197, for the mouse and human counterpart, respectively) had occurred near the N-terminus of LG3. Mutational analysis indicated that Asp but not Asn is crucial for processing of mouse endorepellin (44), possibly by a specific, yet to be discovered, Asp-N endoproteinase.

The second line of evidence is that an identical proteolytic fragment of ~ 25 kDa, cleaved at the same position as the mouse, was detected in the urine of patients with end-stage renal failure (45). This indicates that the LG3 module is present in human serum at relatively high concentrations since this LG3 was found at concentrations of ~10 mg/L of urine (45). Circulating forms of endorepellin may be involved in the homeostatic control of angiogenesis as proposed previously for endostatin, whose levels can reach 0.3 mg/L of blood (36).

Recent experimental tests on tumor-bearing animals are encouraging because protein-based inhibitors, such as endostatin, have three major advantages: 1) they can reduce the tumors to a bearable size, 2) they do not induce resistance, and 3) their toxicity is low (41). Endorepellin is a novel, natural inhibitor of angiogeneis and its use in cancer therapy has additional advantages insofar as endorepellin also exerts an anti-adhesive action on certain tumor cells. The invention disclosed herein presents a method wherein endorepellin inhibits angiogenesis and is used in the treatment of angiogenesis-mediated diseases (*supra*), such as cancer.

Endorepellin also is used in conjunction with standard therapeutic agents to treat angiogenesis-dependent diseases, thereby enhancing the efficacy of current methodologies.

The foregoing descriptions of specific embodiments of the present invention have been presented for the purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention and various embodiments with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto and their equivalents.

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